Cebon et al. Supplementary Methods, Tables and Figures

Supplementary Methods

IFNγ Intracellular Cytokine Staining (ICS)

Expanded NY-ESO-1-specific CD8+ and CD4+) T cells were then assessed by IFNγ intracellular cytokine staining (ICS) using the same peptide pool as well as the individual peptides within the pool. Briefly, CD8+ T cell cultures were stimulated for 4 hours with 1 µM (unless otherwise specified) of peptide in the presence of 10 µg/mL Brefaldin A (Sigma, B7651). Samples were subsequently fixed with 1% paraformaldehyde for 20 min at room temperature and were surface stained with αCD3-PeCy7 and αCD8-PerCP antibodies (BD Bioscience, 341091 or 341050) for 30 mins at 4°C. Cells were permeabilized with 0.3% saponin and intracellularly stained with a αIFNγ-FITC antibody (ThermoFisher, 11-7319-82), initially at room temperature for 30 min, then overnight at 4°C. Samples were analyzed by flow cytometry (FACS Canto II, BD, using Flowjo software, TreeStar) (Supplementary Figure S1B).

Multiplexed Immunofluorescence Analysis

Antigen retrieval was performed in TRS pH 9.0 buffer (Dako, S2367) for NY-ESO-1 detection, whilst citrate pH 6.0 buffer (ThermoScientific, 00-5000) was used for HLA-1. Mouse anti-human HLA-1 (clone HC-10) was used at 1:3000 dilution and mouse anti-human NY-ESO-1 (clone E978) was used at 0.01 µg/mL. Both were incubated for 1 h at room temperature prior to washing and incubation with anti-rabbit-HRP-polymer (DAKO, K4002), and detection with a 1:50 dilution of OPAL-540 TSA (PerkinElmer, NEL796001KT) for HLA-1, or OPAL-520 TSA (NEL796001KT) for NY-ESO-1. Sections were counter stained with spectral DAPI (PerkinElmer, NEL796001KT) and fixed in Vectashield Hard Set Medium (Abacus, H-1400).

Notes & Legends to Supplementary figures

Supplementary Figure S1. Immune monitoring assay schema. (A) Blood sampling timeline for NY-ESO-1 immunity and antigen-specific T-cell assays. (B) NY-ESO-1-specific T cell assay plan.

Supplementary Figure S2: Patient 013-003 demonstrated robust anti-NY-ESO-1 antibody induction within 2 months of vaccination, first detected on day 71 and persisting throughout the 1.5 years of follow-up (Supplementary Figure S2A). Vaccination did not affect other immune reactivities in this patient since $CD8^+$ T cell responses to a control viral peptide pool (FEC) was largely unchanged (Figure S2B). When the antigen-specific T cell response to NY-ESO-1 was assessed after an initial *in vitro* antigen-sensitization (12 day *in vitro* culture with pooled 18mer NY-ESO-1 peptides), there were $CD8^+$ T cell responses specific to peptides 55-73 and 97-115, and $CD4^+$ T cell responses to 103-121, 109-127 and a few other peptides including 121-139. Such responses were also detected when the peptide pool covering the whole NY-ESO-1 sequence (1-180) was used as antigen (Figure S2C). Importantly, these $CD8^+$ and $CD4^+$ T cell responses were not detected prior to vaccination, indicating that the responses were treatment-induced. The response specific to peptides 55-73 was most likely due to the reported previously immunodominant response HLA-B*0702/NY-ESO-1_{60-72} (41).

Supplementary Figure S3: Patient 014-001 demonstrates pre-existing antibody, CD8 and CD4 responses to NY-ESO-1 and the subsequent development of new CD4 specificities following vaccination. (A) Anti-NY-ESO-1 antibody titers after initial vaccination. (B) Internal memory CD8+ T cell response to the control viral peptide pool (FEC). (C) CD8+ and CD4+ T cell responses specific to NY-ESO-1 peptides.

Supplementary Figure S4; Patient 014-005 developed both an antibody and CD4 response following vaccination without any apparent CD8 response. (A) Anti-NY-ESO-1 antibody induction after initial vaccination. (B) Internal memory CD8+ T cell response to a control viral peptide pool (FEC). (C) CD8+ and CD4+ T cell responses specific to NY-ESO-1 peptides

Supplementary Figure S5; Patient 063-002 had induction of multiple CD8 specificities following vaccination which persisted for the entire 1.5 years of follow-up. (A) Anti-NY-ESO-1 antibody titres after initial vaccination. (B) Internal memory CD8+ T cell response to a control viral peptide pool (FEC). (C) CD8+ T cell responses specific to NY-ESO-1 peptides.

Supplementary Figure S6; Patient 014-003 had pre-existing immunity on the basis of prior antibody titres which were not altered following ISCOMATRIX® injections. A $CD4^+$ T cell response did emerge after 6 months. These responses specific to 85-103, 115-133, and potentially other peptide regions, all developed after the fourth adjuvant dose delivered on day 183, two weeks before the blood sampling point at day 197. Notably, the response was short-lived and was not detectable in samples collected prior or later (days 365 and 547). No $CD8^+$ responses were seen over this time course. (A) Anti-NY-ESO-1 antibody titers. (B) Internal memory CD8+ T cell response to a control viral peptide pool (FEC). (C) CD8+ and CD4+ T cell responses specific to NY-ESO-1 peptides.

Supplementary Figure S7; Kinetics and persistence of immune responses. Comprehensive immune monitoring data for all other patients: Vaccine cohort S7.1 - S7.16, ISCOMATRIX® cohort S7.17 - S7.31.

Explanation of slide layout: Panels on the left indicate (i) patient information, (ii) antibody titres, (iii) CD8 IFNg responses to pooled positive control viral peptides derived from: F; influenza, E; Epstein Barr Virus, C; Cytomegalovirus and (iii) interpretation of responses to CD8 and CD4 peptides and antibody.

The panels on the right show IFNγ+ cells in response to overlapping 18-mer peptides for each of the time points tested. The far right bar shows the response to all a pool of all 28 18-mer peptides. Responses >1.0% are shown in boxes.

Centre panels: Illustrative examples of original FACS plots and their interpretation. A positive assay was one in which all controls passed and a population of reactive T cells could be defined on the FACS plot. In most cases, this represented >0.1% IFNγ+ T- cells. Because the assay involves in-vitro pre-stimulation, a positive response should be interpreted as indicating the presence of T cells ex-vivo and not as a quantitative measure (10, 31).

Abbreviations: NT = Not tested, R = response (either v = vaccine-induced response or p = pre-existing, Ab = antibody, NS = No sample, ND = Not determined, ID = patient identifier, d = study day of sample collection, PD progressive disease.

Supplementary Table S1. NY-ESO-1 expression in the populations of screened and eligible patients. Expression was evaluated by IHC and percentages refer to NY-ESO-1 positive cells relative to total number of cells.

Category	Total No. Patients Screened (%)	Total No. Eligible Patients (%)
Negative	381 (61)	20 (14)
<5%	118 (19)	59 (40)
6-25%	41 (7)	19 (13)
26-50%	14 (2)	6(4)
51-75%	15 (2)	11 (7)
>75%	54 (9)	32 (22)
TOTAL	623	147

Supplementary Table S2. Relapse Free Survival during entire period of observation.

Parameter	**NY-ESO-1/ISCOMATRIX® (N=56) n (%)**	**ISCOMATRIX® (N=54) n (%)**	**p-value**
Median Time to Relapse or death (days)	*142*	*176*	*0.398*
Relapse or death			
Yes	33 (58.9)	29 (53.7)	
No	23 (41.1)	25 (46.3)	
Hazard Ratio			0.880
95% CI			(0.532-1.455)
Death due to other causes	3 (5.3)	3 (5.5)	0.234
AJCC Stage Non-IV	*N=38*	*N=37*	
Median Time to Relapse (days)	139	154	0.589
Relapse			
Yes	21 (55.3)	19 (51.4)	
No	17 (44.7)	18 (48.6)	
Hazard Ratio			0.931
95% CI			(0.497-1.745)
AJCC Stage IV	*N=18*	*N=17*	
Median Time to Relapse (days)	215	249	0.468
Relapse			
Yes	12 (66.7)	10 (58.8)	
No	6 (33.3)	7 (41.2)	
Hazard Ratio			0.800
95% CI			(0.346-1.854)

Supplementary Table S3. Vaccine boost and NY-ESO-1 antibody titer increase in patients with or without pre-existing NY-ESO-1 antibodies. Results are shown by study arm, together with a count of relapses occurring in each set of patients.

Study Arm	Pre-existing Ab	Vaccine Boost	No. Patients who Relapsed
NY-ESO-1 + ISCOMATRIX® (N=51)*	Yes (n=16)	Yes (n=9)	4
		No (n=3)	3
		Unknown (n=4)	3
	No (n=31)	Yes (n=28)	14
		No (n=0)	0
		Unknown (n=3)	2
	Unknown (n=4)		1
	Pre-existing Ab	**Ab Titer Increase**	**No. Patients who Relapsed**
ISCOMATRIX® (N=49)*	Yes (n=13)	Yes (n=3)	1
		No (n=9)	6
		Unknown (n=1)	1
	No (n=32)	Yes (n=2)	2
		No (n=26)	16
		Unknown (n=4)	1
	Unknown (n=4)		2

Supplementary Table S4. Summary of NY-ESO-1-specific antibody responses by treatment group in the ITT populations (responses measured by total IgG reciprocal titer). N = total number of patients in each population for whom there are IgG measurements. *n* = number of patients in each population for whom there are IgG measurements at each specific time point. Definition of titers: - = 0-100, + = 100-1,000, ++ = 1,000-10,000, +++ = 10,000-100,000, and ++++ = >100,000. P-values were calculated from a Cochran-Mantel-Haenszel test adjusting for location (UK or Australia/New Zealand).

Titer	NY-ESO-1/ISCOMARIX® (N=52)	ISCOMATRIX® (N=49)	p-value
Baseline	***n =47***	***n = 45***	**0.733**
-	31 (65.9%)	32 (71.1%)	
+	2 (4.2%)	0 (0.0%)	
++	8 (17.0%)	10 (22.2%)	
+++	4 (8.5%)	2 (4.4%)	
++++	2(4.2%)	1 (2.2%)	
Day 71	***n = 42***	***n = 42***	**0.000**
-	0 (0.0%)	29 (69.0%)	
+	0 (0.0%)	3 (7.1%)	
++	2 (4.8%)	7 (23.8%)	
+++	31 (73.8%)	2 (4.8%)	
++++	9 (21.4%)	1 (2.4%)	
Day 197	***n = 34***	***n = 33***	**0.002**
-	0 (0.0%)	21 (63.6%)	
+	0 (0.0%)	0 (0.0%)	
++	3 (8.8%)	7 (21.2%)	
+++	20 (58.8%)	5 (15.1%)	
++++	11 (32.3%)	0 (0.0%)	
Day 365	***n = 27***	***n = 29***	**0.000**
-	0 (0.0%)	19 (65.5%)	
+	1 (3.7%)	0 (0.0%)	
++	2 (7.4%)	6 (20.7%)	
+++	23 (85.2%)	4 (13.8%)	
++++	1 (3.7%)	0 (0.0%)	
End of Study	***n = 37***	***n = 36***	**0.000**
-	0 (0.0%)	26 (72.2%)	
+	2 (5.4%)	2 (5.5%)	
++	6 (16.2%)	4 (11.1%)	
+++	26 (70.3%)	4 (11.1%)	
++++	3 (8.1%)	0 (0.0%)	